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Research Paper

Exposure to drinking-water disinfection byproducts, seminal plasma metabolomic signatures, and semen quality: Findings from the TREE cohort

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ABSTRACT

Background: Disinfection byproducts (DBPs) have been linked to reduced semen quality, yet the underlying mechanisms are largely unclear.
Objective: We investigated seminal plasma metabolomic signatures of exposure to DBPs and semen quality and further explored their mediating roles in associations linking exposure to DBPs with semen quality.
Methods: Untargeted metabolomics analysis and Metabolome-Wide Association Studies (MWAS) were employed to characterize seminal plasma metabolites of DBP exposures and semen quality parameters among 193 participants from the Tongji Reproductive and Environmental (TREE) cohort. Urinary biomarkers of dichloroacetic acid (DCAA) and trichloroacetic acid (TCAA) were assessed as drinking-water DBP exposures.
Results: Negative associations between urinary DCAA concentration and sperm total motility and progressive motility were observed. Among the 812 annotated metabolites, urinary DCAA and TCAA concentrations were separately linked with 28 and 24 metabolites, which were primarily involved in arginine and proline metabolism pathway. Additionally, 232 metabolites were related with semen quality parameters, mainly participating in arginine and proline, aspartate and glutamate, arachidonic acid, alanine, and D-amino acid metabolism pathways. Mediation analyses revealed that phosphatidylinositol [PI(18:1/22:6)] mediated the associations between urinary DCAA concentration and reduced sperm progressive motility and total motility, with the mediating effects of 34% and 31%, respectively.
Conclusions: Our findings provide novel insights into potential biomarkers of exposure to DBPs and semen quality and enhance understanding of the biological mechanisms through which DBP exposures exert adverse effects on semen quality.

1. Introduction

Infertility has been an increasing public health concern worldwide. Approximately 17.5% of couples are influenced by infertility globally, with male factors accounting for roughly half of these cases [1]. Semen quality is an important indicator for assessing male fertility, but accumulating evidence suggests that there has been a declining trend in semen quality [2–4]. The latest meta-analysis indicates that total sperm count and sperm concentration decrease by 62.3% and 51.6% separately from 1973 to 2018 [5]. In China, a systematic review also reports the decline of 28.13% in total sperm count and 36.95% in sperm concentration among healthy Chinese men from 1981 to 2019 [6]. Several risk

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factors have been linked to the decline of semen quality, including dietary changes, unhealthy lifestyles, and exposure to environmental chemicals [7–9].

Disinfection byproducts (DBPs) are the deleterious chemical compounds that arise from the interaction between disinfectants and organic substances within the disinfection process of drinking water treatment [10]. To date, over 2000 distinct DBPs have been found, resulting in ubiquitous human exposures via inhalation, dermal absorption, and ingestion of DBPs [11,12]. Human biomonitoring has documented that DBPs can be detected in multiple biological samples such as blood, urine, and exhaled breath [13–15]. Studies have also demonstrated that DBPs can pass through blood-testis barrier to the testicular environment [16], which can cause exert adverse effects on male reproductive function. Numerous toxicological researches indicate that DBP exposures induce pathological alterations, diminish testicular weight, lower testosterone levels, and impair spermatogenesis [17–21]. Consistently, our previous epidemiological studies have also revealed the deterioration of semen quality in relation to DBP exposures measured by blood and urinary biomarkers [22–24]. However, the underlying biological mechanisms remains largely unclear.

Metabolomics serves as a sensitive analytical tool for delineating alterations in metabolites and molecular pathways consequent to environmental exposures, which offers the potential to uncover the biological responses to exposures and the mechanisms of toxicity [25]. To our best knowledge, only two human studies to date have investigated serum metabolomic signatures associated with exposure to DBPs [26,27]. These studies suggest that DBP exposures potentially impacts the tryptophan and tyrosine metabolic pathways. However, these serum-based metabolomic studies primarily reflect the systemic metabolic alterations, lacking specificity for the male reproductive system—they cannot directly link DBP exposures to the local microenvironment relevant to sperm function. In contrast to serum, seminal plasma is the microenvironment for sperm development and function, which has been regarded as a promising biological specimen for metabolomic analysis in assessing semen quality [28,29]. Significant differences in seminal plasma metabolites have been documented between men with oligospermia or asthenozoospermia and those with normal semen quality [30–32]. However, the seminal plasma metabolite profiles of exposure to DBPs have not yet been evaluated. Furthermore, no studies have explored the potential mediating effects of seminal plasma metabolic changes in connecting DBP exposures with semen quality.

In light of the knowledge gaps, we in the current study utilized an untargeted metabolomics analysis to characterize seminal plasma metabolites linked with DBP exposures and semen quality, and to further explore their mediating roles in the pathway linking DBP exposures with semen quality. Urinary dichloroacetic acid (DCAA) and trichloroacetic acid (TCAA) concentrations were measured as internal biomarkers to reflect drinking-water DBP exposures [33,34]. The use of seminal plasma as a target matrix for metabolomics analysis would specifically provide novel mechanistic insights into the male reproductive toxicity of DBPs.

2. Methods

2.1. Study population

This study was based on the Tongji Reproductive and Environmental (TREE) cohort established between December 2018 and August 2021 in Wuhan, Hubei, China, which has been detailed elsewhere [22,35]. In brief, couples who firstly visited the Wuhan Tongji Reproductive Medical Center for assisted reproductive technology treatment were enrolled into the TREE cohort. The couples went through health testing at enrollment, followed by gamete collection, and pregnancy examination A total of 2784 male partners were recruited. Men with occupational exposure to specific chemicals such as trichloroethylene and dichloroethylene related to potential exposure sources of DCAA and TCAA or certain reproductive diseases such as epididymitis and varicocele associated with poor semen quality were anteriorly excluded. After that, a subpopulation of 200 male partners of couples who lived in Wuhan and also provided both complete questionnaires and semen samples were selected to measure seminal plasma metabolomics, as outlined in our earlier research [36]. Among them, 7 men had no available urinary exposure assessment, resulting in 193 participants included into the current analysis. Except for age, education, and household income, no significant differences occurred on sociodemographic characteristics between this included subjects and those of all recruited men (Table S1). The Ethics Committee of Tongji Medical College approved the study protocol (ID: No. 2019-S004), and all participants signed informed consent.

2.2. Urine collection and measurements

Urine specimens were collected in 50-mL polypropylene containers on the day of enrollment (first visiting day to the clinic for health testing) and then were portioned and stored at −20 °C in the laboratory. Specific methods for quantification of urinary DCAA and TCAA levels have been described in detail in previous studies [37]. Briefly, a 5-mL urine specimen was mixed sequentially with 1-mL concentrated sulfuric acid, 5 g of anhydrous sodium sulfate, and 1.5 g of anhydrous copper sulfate. Then the sample was extracted using 5-mL extraction solution containing an internal standard (1,2-dipropyl bromide). After centrifugation, 3-mL supernatant was separated and reacted with 1-mL acidified methanol for 2 h in a 50 °C water bath. The reaction was halted by adding a saturated sodium bicarbonate solution. Finally, the target analytes were detected via gas chromatography.

One blank and two additional quality control (QC) samples were measured alongside each batch. The limits of detection (LODs) were 1.00 μg/L for DCAA and 0.50 μg/L for TCAA. Values below the LODs were substituted with $\mathrm{LOD}/\sqrt{2}$ for analysis. The relative standard deviations for intraday and interday measurements were less than 10.00%, and the spiked recoveries of urinary samples ranged from 92.00% to 118.19%. The urinary specific gravity (SG) was measured via a handheld digital refractometer. Urine dilution was corrected with the formula: $Hs = H \times [(1.018 - 1) / (SGc - 1)]$. The Hs and H represent SG-adjusted and unadjusted urinary target concentrations, respectively. SGc is the SG concentration for each sample, and 1.018 is the mean SG concentration for participants in this study.

2.3. Semen collection and analysis

Participants were asked to provide a single semen sample by masturbation into a sterile polypropylene container in a designated room during gamete collection, with an average interval of 72 days from urine sample collection, which corresponds approximate one sperm development cycle. The sample was kept at 37 °C to facilitate liquefaction and then was analyzed within 60 min by specialized laboratory technicians, in accordance with the guidelines from the World Health Organization (WHO), as previously outlined [22,23,38]. In brief, the volume of the semen was ascertained by weighing the sample. Subsequently, sperm motility and concentration were assessed using a computer-aided sperm analysis system. Sperm count was obtained by multiplying semen volume by sperm concentration. Total motility was determined by summing the percentages of progressive and non-progressive motility.

2.4. Metabolomic analysis

After evaluating the semen quality, the residual semen samples underwent a process of separation and centrifugation to obtain seminal plasma. The seminal plasma was subsequently stored at −80 °C for metabolite analysis, as previously detailed [36]. In short, a 100 μL of the

seminal plasma sample was transferred into a tube, and 300 μL of extraction solution containing methanol and isotopically labeled internal standards was added. The mixture was vortexed and sonicated in an ice-water bath, followed by protein precipitation. The supernatant was isolated via centrifugation and transferred to a new glass vial for further analysis. An equal mixture of the supernatant from all seminal plasma samples was created as a QC sample.

Seminal plasma metabolites were detected via liquid chromatography-mass spectrometry (LC-MS/MS) with a reverse-phase UPLC HSS T3 column and an Orbitrap Exploris 120 mass spectrometer. Electrospray ionization (ESI) was conducted in both positive and negative modes, and MS/MS spectra were collected in information-dependent acquisition (IDA) mode. The optimized LC-MS/MS parameters and data acquisition have been detailed in our previous study [36]. Raw data were transformed to mzXML format using ProteoWizard and then handled in R with the "XCMS" package for peak identification, alignment (by matching mass-to-charge ratio and retention time), and integration (based on peak area calculation) [39]. The peaks with a coefficient of variation (CV) exceeding 30% in QC samples were removed. Finally, 22135 peaks were normalized based on the corresponding internal standard peak areas. The metabolites were identified by matching secondary mass spectrometry (MS2) spectra against BiotreeDB, an internal MS2 database, and the Human Metabolome Database (HMDB).

2.5. Covariates

Sociodemographic information was collected via a questionnaire at enrollment, including household income, educational background, alcohol consumption, smoking history, and occupational exposures. Body mass index (BMI, kg/m^2) was determined from weight and height measurements during clinic visits, calculated as weight (kg) divided by height squared (m). Abstinence time (days) and age (years) were abstracted from the medical record. Subjects were classified as smokers if they reported a lifetime history of smoking 100 or more cigarettes [40]. Individuals who reported drinking alcohol more than once a week for at least 6 months were classified as alcohol consumers [41].

2.6. Statistical analysis

R software (version 4.3.1) was utilized for data analysis. Descriptive statistical analysis was conducted on the basic characteristics, as well as the distribution of semen quality parameters and urinary DCAA and TCAA concentrations. Pearson's correlation coefficient was utilized to investigate correlations between metabolites. Urinary DCAA and TCAA concentrations, semen quality parameters, and metabolite intensities were subjected to natural logarithmic transformation to meet the assumptions required for the further analysis.

Linear regression models were employed to assess the associations between urinary DCAA and TCAA concentrations (exposures) and semen quality parameters (outcomes). In these models, the exposures were treated as both continuous and categorical variables. Linear trend tests were performed by designating the tertiles of exposures (T1-T3) as ordinal variables (1–3). The results were presented as percentage changes (%), calculated for each one-unit increase in continuous exposure variables (percentage change = 100% × [exp(beta × ln(2)) − 1]), or as percentage changes comparing the upper tertiles to the lowest tertiles for categorical exposure variables (percentage change = 100% × [exp(beta) − 1]).

Metabolome-Wide Association Studies (MWAS) were utilized to identify metabolites associated with DBP exposures and semen quality parameters, employing a stepwise linear regression analysis. Initially, univariate linear regression models were used to examine the associations of each metabolite with exposures or outcomes. Metabolites with p-value <0.05 in the univariate linear regression models were selected as candidates for subsequent validation. Subsequently, multivariate linear regression models were applied to validate these candidate metabolites, with potential confounders adjusted. To address multiple comparisons and the false discovery rate (FDR), the Benjamini-Hochberg (BH) method was applied to adjust the p-value. The metabolites with FDR-adjusted p-value ≤0.05 were considered significantly associated with DBP exposures or semen quality parameters.

Covariates were selected based on prior studies reporting to be related with DBP exposures and semen quality parameters [22,24,26,27,42] and then identified by directed acyclic graph (Fig. S1). Finally, abstinence time, age, and BMI were included as continuous variables; alcohol use (never, current and former) and smoking status (never, current and former) were included as categorical variables; household income (≤ 3000, 3001–5000, 5001–10,000, and > 10,000 yuan per month) and education level (less than high school, high school, and more than high school) were included as ordered categorical variables. Given that physical activity may affect DBP exposures or semen quality, we additionally performed a sensitivity analysis with adjustment for physical activity.

Pathway analysis was conducted using the MetaboAnalyst platform (https://new.metaboanalyst.ca/home.xhtml), a widely utilized online tool for metabolomics data analysis. Meet-in-the-middle (MITM) approach was used to identify significant overlapping metabolites and pathways associated with both DBP exposures and semen quality parameters. Furthermore, mediation analysis was utilized to assess the potential mediating effects of overlapping seminal plasma metabolites on the relationship between DBP exposures and semen quality parameters. Candidate metabolites meeting the following assumptions were included in the linear mediation model: (1) significant linear association between exposure and mediator; (2) significant linear association between mediator and outcome; (3) no exposure-mediator interaction. The direct effect reflects the relationship between DBP exposures and semen quality parameters without the involvement of intermediate metabolites. The indirect effect assesses how DBP exposures influences semen quality parameters via potential seminal plasma metabolites. The total effect of DBP exposures on semen quality parameters encompasses both direct and indirect effects. The mediation proportion was calculated using the formula: mediation proportion = (indirect effect/total effect) × 100%. The R package "mediation" was used for the mediation analyses according to the quasi-Bayesian Monte Carlo method with 1000 simulations and incorporated the same covariates as above.

3. Results

Table 1 outlines the study population's characteristics. The average (SD) age, BMI, and abstinence time were 33.38 (5.01) years, 24.62 (2.85) kg/m^2, and 4.61 (4.44) days, respectively. Among the included 193 subjects, 130 men (67.36%) had over high school education levels, and 67 men (34.72%) reported a monthly household income of 5001–10,000 RMB. Additionally, 92 men (47.66%) were identified as never-smokers and 154 men (79.79%) as never-drinkers. The arithmetic means of sperm total motility, progressive motility, count, and concentration were 41.56%, 39.12%, 142.20 × 10^6 /ejaculate, and 49.25 × 10^6 /mL, respectively. For urinary DCAA and TCAA, the arithmetic means of SG-adjusted concentrations were 5.98 and 5.69 μg/L, respectively (Table 2).

A total of 812 metabolites were detected via MS2 annotation in seminal plasma metabolomic profiling. Univariate linear regression analysis revealed that 60 metabolites were associated with urinary DCAA and TCAA concentrations ($p < 0.05$), while 279 metabolites were associated with semen quality parameters ($p < 0.05$) (Fig. 1A and Table S2). Multivariate linear regression analysis identified 45 metabolites that were associated with at least one type of urinary DCAA and TCAA concentrations, and 232 metabolites that were associated with at least one semen quality parameter (FDR ≤ 0.05) (Fig. 1B). These results remained essentially unchanged after adjustment for physical activity (Table S12–14).

Table 1
Characteristics of the study participants ($N = 193$).

Characteristics	Mean ± SD or n (%)
Abstinence time (days) [a]	4.61 ± 4.44
<3	12 (6.35)
3–5	157 (83.07)
>5	20 (10.58)
Age (years old)	33.38 ± 5.01
≤30	57 (29.53)
>30	136 (70.47)
BMI (kg/m^2)	24.62 ± 2.85
≤24.0	80 (41.45)
>24.0	113 (58.55)
Educational level	
Less than high school	25 (12.95)
High school	38 (19.69)
More than high school	130 (67.36)
Household income (yuan/month)	
≤ 3000	12 (6.22)
3001–5000	48 (24.87)
5001–10,000	67 (34.72)
>10,000	66 (34.19)
Smoke status	
Never	92 (47.66)
Current	73 (37.82)
Former	28 (14.51)
Alcohol status	
Never	154 (79.79)
Current	17 (8.81)
Former	22 (11.40)

Abbreviation: SD: standard deviation; BMI: body mass index.
[a] A total of 4 men missing abstinence time.

Fig. 2A and Table S3 illustrates the associations between urinary DCAA concentration and 28 metabolites, which are primarily categorized into five classes: Lipids and lipid-like molecules (71.4%), Benzenoids (10.7%), Organic acids and derivatives (7.1%), Phenylpropanoids and polyketides (7.1%), and Organic nitrogen compounds (3.6%). Twenty-four metabolites were associated with urinary TCAA concentration (Fig. 2B and Table S4), spanning across five classes: Lipids and lipid-like molecules (54.2%), Organic acids and derivatives (16.7%), Benzenoids (8.2%), Phenylpropanoids and polyketides (4.2%), and Others (16.7%). Interestingly, 7 metabolites were negatively associated with both urinary DCAA and TCAA concentrations (Fig. 2D) and were also found to be positively intercorrelated (Fig. 2E). Pathway analysis showed that arginine and proline metabolic pathway was significantly associated with urinary TCAA concentration (Fig. 2C and Table S7).

Results displayed in Fig. 3 show the relationships between metabolites and semen quality parameters. Specifically, 86 and 146 metabolites were positively and negatively associated with four semen quality parameters, respectively (Fig. 3A). Venn analysis revealed that 30 metabolites were associated with all four semen parameters (Fig. 3C). These metabolites predominantly belong to five categories: Lipids and lipid-like molecules (63.3%), Organic oxygen compounds (6.7%), Phenylpropanoids and polyketides (10%), Organic acids and derivatives (13.3%), and Others (6.7%). Of the 30 metabolites identified, 26 metabolites were positively associated with the four semen quality parameters, while the remaining 4 metabolites (6-Hydroxydaidzein, THP-4 A, Aspartyl-Valine, and Aspartyl-Isoleucine) exhibited negative associations (Fig. 3D, Tables S5 and S6). Pathway enrichment analysis revealed that arginine and proline metabolism, D-amino acid metabolism, alanine, aspartate and glutamate metabolism, and arachidonic acid metabolism pathways were related with semen quality parameters (Fig. 3B and Table S7).

In this subpopulation of longitudinal analysis, we still found inverse associations with sperm progressive motility and total motility for elevated tertiles of urinary DCAA levels (all p for trends < 0.05) (Table S10). Using MITM approach, we identified one overlapping pathway (arginine and proline metabolism) and nine overlapping metabolites. In the arginine and proline metabolic pathway, urinary TCAA concentration was negatively associated with 4-aminobutyraldehyde and spermine. Regarding semen quality parameters, sperm concentration showed a negative association with L-proline and pyruvic acid, but a positive association with D-proline. Additionally, sperm count was positively associated with L-glutamic acid (Fig. 4).

As shown in Fig. 5, nine metabolites were associated with both DBP exposures and semen quality parameters. These metabolites included lipids and lipid-like molecules such as phosphatidylglycerol [PG(18:1/18:2), PG(22:5/22:5)], lyso-phosphatidylethanolamine [LysoPE(22:6/0:0)], phosphatidylinositol [PI(18:1/22:6)], lyso-phosphatidylserines [LPS(22:5)], and acyl carnitines [ACar(17:0)], as well as organic acids and derivatives such as Aspartyl-Valine, Leucyl-Lysine, and Malonic acid (Fig. 5A, Tables S8 and S9). Notably, PI(18:1/22:6) was negatively associated with urinary DCAA (−23.53, 95% CI: −36.19, −8.35) and TCAA (−14.56, 95% CI: −26.11, −1.20) concentrations, and also were positively associated with sperm concentration (14.42, 95% CI: 2.87, 27.26), sperm count (13.72, 95% CI: 1.11, 27.90), sperm progressive motility (11.07, 95% CI: 3.16, 19.58), and sperm total motility (10.06, 95% CI: 2.77, 17.88) (Fig. 5B). The results of mediation analysis showed that PI(18:1/22:6) played the potential mediating roles in the reduction of sperm progressive motility and total motility caused by urinary DCAA, with the mediating effects of 34% and 31%, respectively (Fig. 5C and Table S11).

4. Discussion

Among this reproductive-aged Chinese men from the TREE cohort, we investigated the seminal plasma metabolomic signatures of DBP exposures, measured via urinary DCAA and TCAA. We found that 45 metabolites were associated with DBP exposures, predominantly implicated in arginine and proline metabolism pathway. Additionally, 232 metabolites were associated with semen quality parameters, mainly participating in arginine and proline metabolism, D-amino acid

Table 2
Distribution of semen parameters and urinary DCAA and TCAA concentrations from study participants (N = 193).

Variables	Arithmetic mean	Geometric mean	5th	25th	50th	75th	95th	min	max
Semen parameters									
Sperm concentration (10^6 per mL)	49.25	38.57	10.60	26.00	41.00	68.00	106.40	4.00	233.00
Sperm count (10^6 per ejaculate)	142.20	105.67	24.60	63.00	120.00	184.00	356.80	7.00	546.00
Sperm progressive motility (%)	39.12	34.95	13.00	27.00	39.00	51.00	65.00	3.00	72.00
Sperm total motility (%)	41.56	37.63	15.00	30.00	42.00	53.00	68.00	4.00	76.00
Urinary DCAA and TCAA									
Unadjusted (μg/L)									
DCAA	5.28	4.97	2.66	4.11	5.15	6.34	8.27	1.24	17.03
TCAA	5.32	4.57	1.61	3.25	4.78	6.41	10.79	0.53	22.14
SG-adjusted (μg/L)									
DCAA	5.98	5.38	3.16	4.25	4.93	6.29	13.02	2.64	30.45
TCAA	5.69	4.95	2.28	3.46	4.66	6.29	13.85	1.73	20.69

Abbreviation: DCAA: dichloroacetic acid; SG: specific gravity; TCAA: trichloroacetic acid.

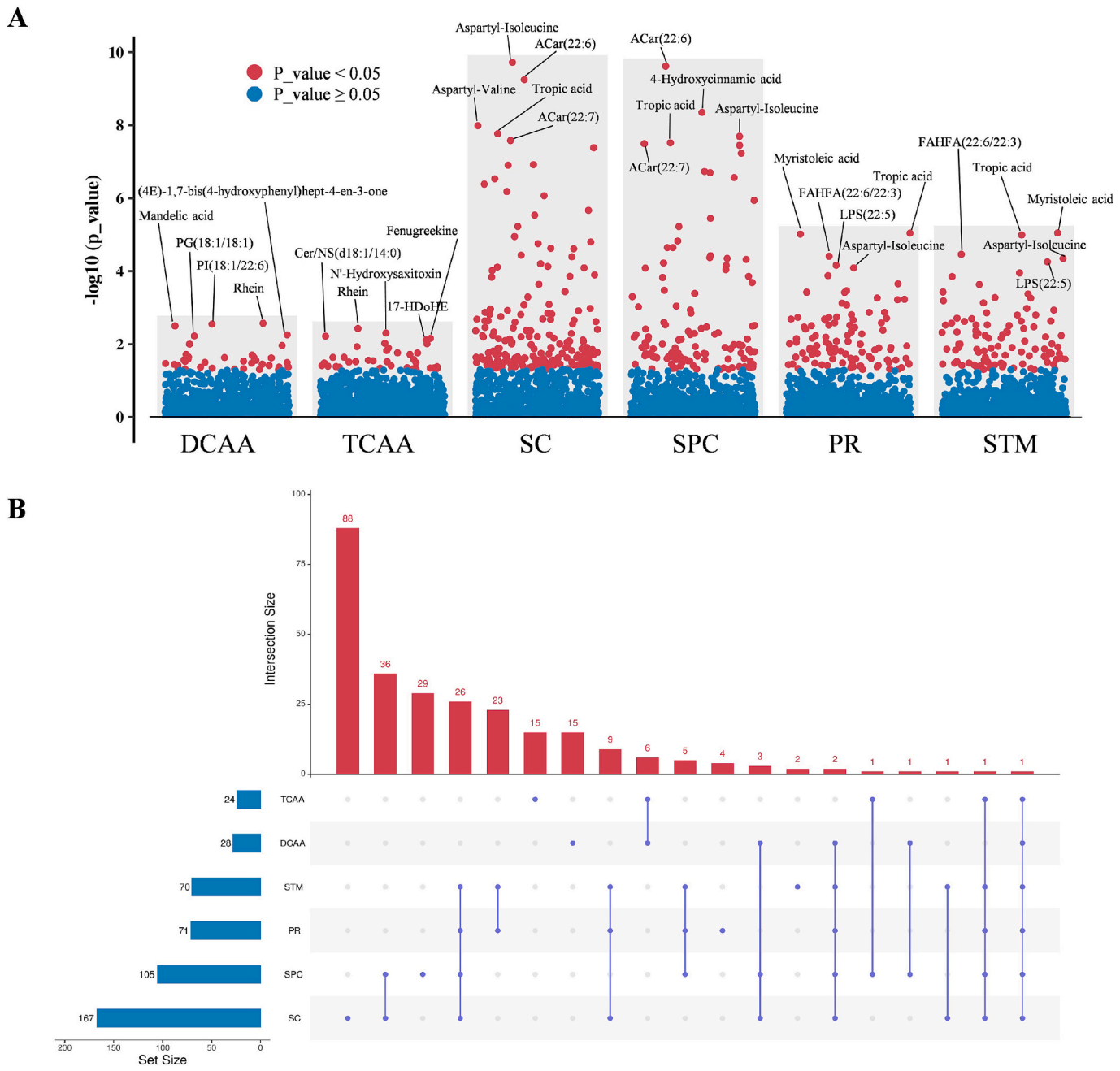

Fig. 1. Associations of seminal plasma metabolites with DBP exposures or semen quality parameters. (A) Manhattan plot showing the significance of the associations of seminal plasma metabolites with DBP exposures or semen quality parameters. (B) Upset plot showing the number of seminal plasma metabolites significantly associated with DBP exposures or semen quality parameters. Models were adjusted for age, BMI, abstinence time, smoking status, alcohol use, education level, and household income, and all *p*-values were adjusted for multiple comparisons using the Benjamini-Hochberg FDR method, with a FDR of 0.05 as a statistically significant association. Abbreviation: DCAA: dichloroacetic acid; DBPs: Disinfection byproducts; TCAA: trichloroacetic acid; SC: sperm concentration; SPC: sperm count; PR: sperm progressive motility; STM: sperm total motility.

metabolism, alanine, aspartate and glutamate metabolism, and arachidonic acid metabolism pathways. Nine metabolites were found to be related with both DBP exposures and semen quality parameters. Furthermore, PI(18:1/22:6) was identified as a potential mediator in the relationships between urinary DCAA concentration and both diminished sperm progressive motility and total motility.

To our knowledge, this study is the first one to characterize seminal plasma metabolites of exposure to DBPs measured by urinary biomarkers. We found that 28 and 24 seminal plasma metabolites were separately associated with urinary DCAA and TCAA concentrations. Furthermore, pathway analysis indicated that those metabolites were significantly enriched in arginine and proline metabolic pathway. Two previous human studies have explored the serum metabolomic signatures associated with exposure to DBPs. A case-control study of exposure to drinking-water trihalomethanes (THMs) and colorectal cancer identified 21, 20, and 20 serum features associated with total THMs, brominated-THMs, and chloroform, respectively; pathway analysis suggested that these features were involved in the complexine metabolic pathway and the arginine and proline metabolic pathway [27]. Kogevinas et al. investigated metabolic changes in blood samples from 60 volunteers before and after swimming in a chlorinated pool; they identified 293 metabolic features related with at least one DBPs in

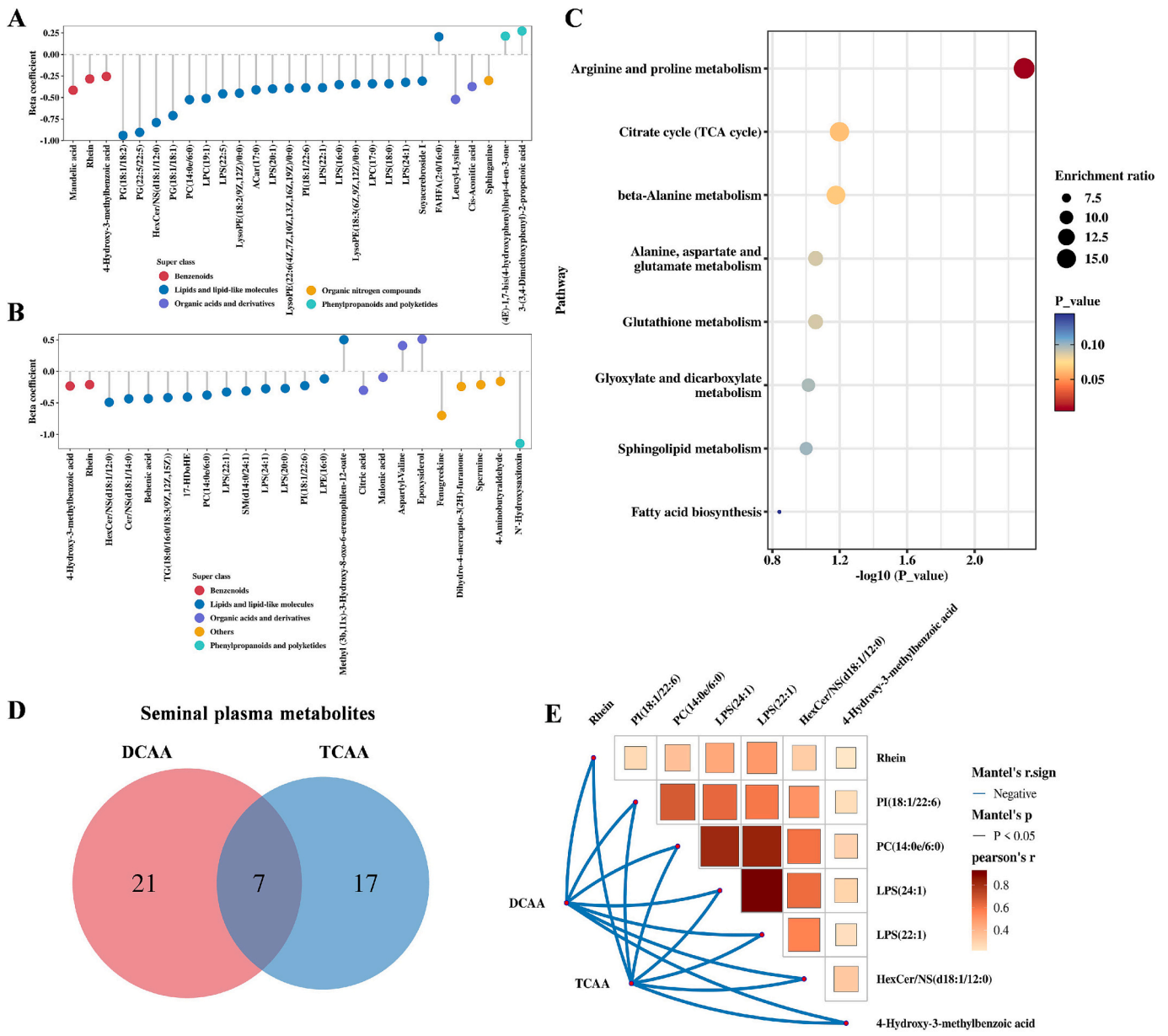

Fig. 2. Associations and pathway enrichment of seminal plasma metabolites with DBP exposures. (A and B) Lollipop plots display beta coefficients of metabolites significantly associated with urinary DCAA (A) or TCAA (B) concentrations. The color of the dots indicates the super-class of metabolites. Beta coefficients represent the percentage change in metabolite intensity per 1% increase in urinary DCAA or TCAA concentrations. Models were adjusted for age, BMI, abstinence time, smoking status, alcohol use, education level, and household income. (C) Bubble plot showing metabolic pathways enriched by metabolites significantly associated with urinary DCAA and TCAA concentrations. (D) Venn plot showing the number of overlapping metabolites significantly associated with both urinary DCAA and TCAA concentrations. (E) Correlation network heatmap between DBPs, metabolites, and inter-metabolite relationships. Abbreviation: DCAA: dichloroacetic acid; DBPs: Disinfection byproducts; TCAA: trichloroacetic acid.

exhaled breath, which were significantly enriched in the tryptophan metabolic pathway [26]. Specially, this study identified 18 metabolic features associated with urinary TCAA, but this association disappeared after adjusting for energy expenditure [26]. A targeted metabolomics analysis of *Daphnia magna* also found that DCAA and TCAA exposures resulted in significant decreases in specific metabolites such as adenosine, guanosine, and inosine [43]. These results suggest that dysregulated metabolic pathways may be potential biological mechanisms of DBP exposures leading to toxic damage.

Notably, among the metabolites we found to be associated with DBP exposures, some newly metabolites (mainly lipids and lipid-like molecules) were also identified, though they were not amenable to pathway analysis. Lipids are not only the major components of cell membranes but also play crucial roles in energy storage, signaling, and maintaining cell function and overall health [44]. Toxicological studies found that DBP exposures induced abnormal lipid metabolism and exacerbated lipid aggregation in the liver and serum of mice [45]. Zhang et al. found that DBP exposures induced disturbances in amino acid metabolism and lipid metabolism to cause toxic injury in mice by metabolomics analysis [46].

Numerous studies have demonstrated altered seminal plasma metabolites in association with semen quality [47,48]. Our study identified 232 metabolites associated with at least one semen quality parameter, with 30 metabolites associated with all four semen quality parameters. Specifically, lipid metabolites (such as FAHFA(18:2/20:4), PI(18:1/22:6)), acylcarnitine metabolites (such as ACar(18:1), ACar(13:0)), and

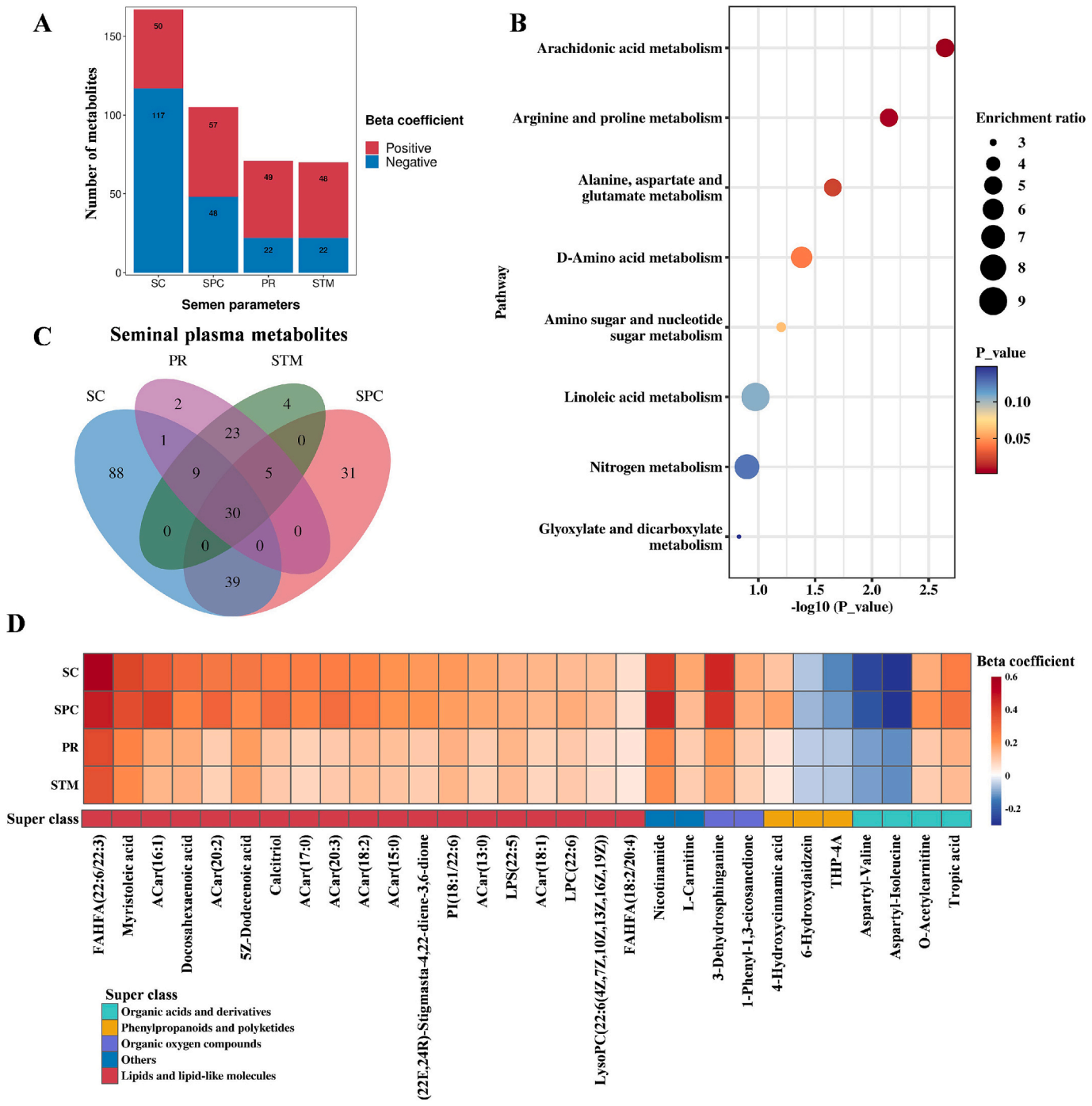

Fig. 3. Associations and pathway enrichment of seminal plasma metabolites with semen quality parameters. (A) Bar plot showing the number of metabolites significantly associated with semen quality parameters. Beta coefficients represent the percentage change in semen quality parameters per 1% increase in metabolite intensity. Models were adjusted for age, BMI, abstinence time, smoking status, alcohol use, education level, and household income. (B) Bubble plot showing metabolic pathways enriched for metabolites significantly associated with semen quality parameters. (C) Venn plot showing the number of shared and unique metabolites significantly associated with the four semen quality parameters. (D) Heatmap of beta coefficients for metabolites significantly associated with four semen quality parameters. Abbreviation: SC: sperm concentration; SPC: sperm count; PR: sperm progressive motility; STM: sperm total motility.

polyunsaturated fatty acids (such as docosahexaenoic acid) were positively associated with semen quality parameters. These metabolites enhance sperm motility, morphology, and function via multiple mechanisms, including energy metabolism, antioxidant activity, membrane fluidity, and cell signaling pathways [49–51]. For instance, lipid metabolites contribute to sperm motility by engaging in fatty acid β-oxidation and the tricarboxylic acid cycle to generate ATP, which supplies the necessary energy for sperm function [52]. Moreover, four metabolites (6-Hydroxydaidzein, THP-4A, Aspartyl-Valine, and Aspartyl-Isoleucine) were negatively associated with semen quality parameters. For example, 6-Hydroxydaidzein, an isoflavone compound, has been shown to reduce insulin sensitivity in mouse adipocytes, which may adversely affect sperm energy metabolism and function [53]. Pathway analysis revealed these altered seminal plasma metabolites were involved in several key metabolic pathways, including arachidonic acid metabolism, arginine and proline metabolism, alanine, aspartate

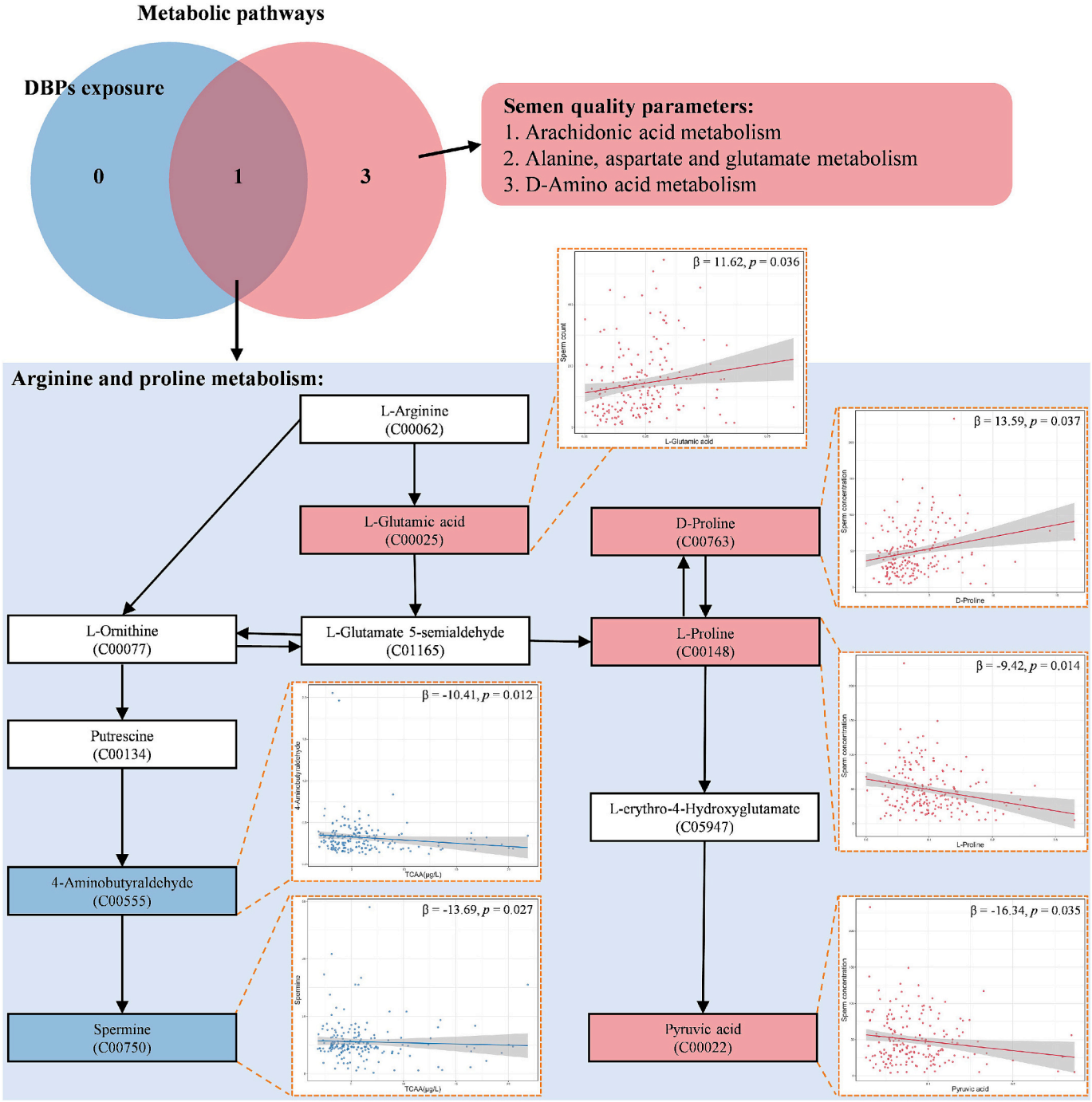

Fig. 4. Metabolic pathways related to DBP exposures and semen quality parameters. The boxes in the figure display some core metabolites in the arginine and proline metabolic pathway. Blue boxes indicate metabolites associated with DBP exposures, red boxes indicate metabolites associated with semen quality parameters, and white boxes represent metabolites with no identified associations. The scatter plots illustrate the associations between seminal plasma metabolites and either DBP exposures or semen quality parameters. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and glutamate metabolism, and D-amino acid metabolism. In support of our findings, prior studies have identified that abnormalities in these pathways such as alterations in arachidonic acid metabolism and arginine and proline metabolism are closely related to semen quality and male fertility [36,54,55].

In this study, the arginine and proline metabolism pathway was found to be the only overlapping metabolic pathway associated with DBP exposures and semen quality parameters. Proline is the preferred energy substrate for mature spermatozoa, which lack a complete cytoplasmic glycolysis system and mainly rely on proline dehydrogenase (PRODH) to catalyze proline oxidation for ATP production required for sperm tail movement and the acrosome reaction [56–58]. Arginine serves as the rate-limiting precursor for endogenous nitric oxide (NO) synthesis [59]; NO generated by nitric oxide synthase (NOS) scavenges excessive reactive oxygen species (ROS) in the reproductive microenvironment and regulates sperm membrane ion channels to mediate capacitation and the acrosome reaction [60,61]. Accordingly, numerous experimental studies have demonstrated that DBP exposures induce intracellular ROS overaccumulation [62–64]. Our previous human studies have also shown that urinary DCAA and TCAA were positively

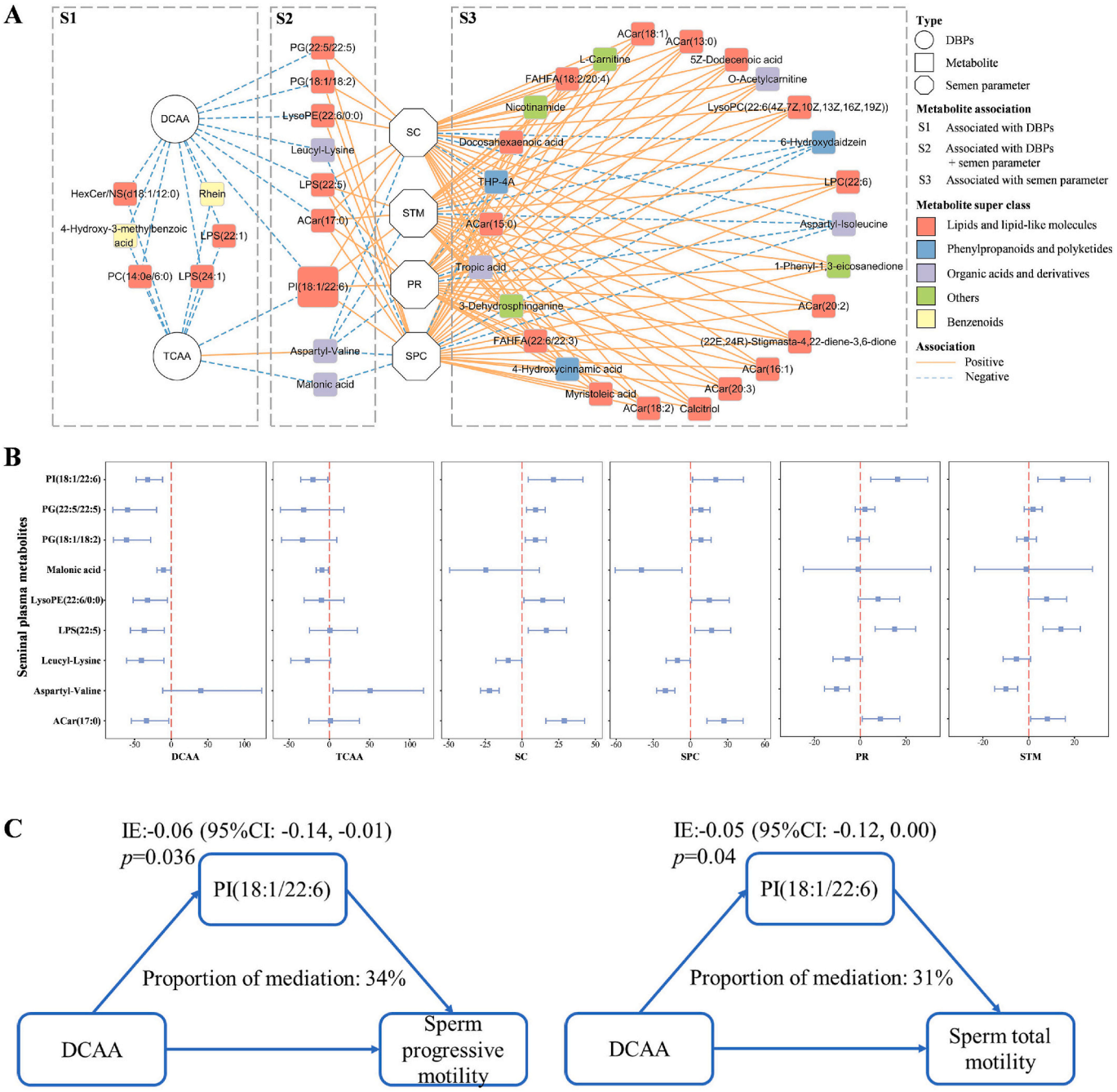

Fig. 5. The network and mediation analysis of seminal plasma metabolites, DBP exposures, and semen quality parameters. (A) Correlation network of metabolites with DBP exposures or semen quality parameters. (B) Percentage change in the associations of metabolites with DBP exposures or semen quality parameters. Percent change (%) = 100% × [exp(beta × ln(2)) − 1]. (C) Mediating effect of metabolites in the associations between DBP exposures and sperm progressive motility or total motility. Abbreviation: DCAA: dichloroacetic acid; DBPs: Disinfection byproducts; TCAA: trichloroacetic acid; SC: sperm concentration; SPC: sperm count; PR: sperm progressive motility; STM: sperm total motility; IE: indirect effect.

associated with oxidative stress biomarkers such as 8-hydroxy-2-deoxyguanosine (8-OHdG) and 8-isoprostaglandin $F_{2\alpha}$ (8-isoPGF$_{2\alpha}$) [65,66]. The increasing oxidative stress levels induced by exposure to DBPs may directly or indirectly affect the arginine-proline metabolic pathway.

Toxicological studies have documented that DBP exposures induce diminished semen quality [17,67]. Consistent with the experimental evidence, our previous epidemiological surveys have found the associations between DBP exposures measured by blood THMs and urinary DCAA or TCAA and reduced semen quality parameters [22–24,42,66,68]. However, the causal relationships are warranted to further confirm. In this subpopulation of 193 men with longitudinal analysis, urinary DCAA in negative associations with sperm total motility and progressive motility were still observed. We further elucidated the mediating role of seminal plasma metabolites underlying the associations between DBP exposures and reduced semen quality. We identified 9 metabolites associated with both DBP exposures and semen quality parameters. Furthermore, mediation analysis revealed that PI (18:1/22:6) acts as a potential mediator between urinary DCAA concentration and both reduced progressive motility and total motility. Previous studies have shown that PI(18:1/18:1) can inhibit the activation of p38 MAPK, thereby reducing stress responses such as apoptosis and autophagy and maintaining cell morphology and proliferation [69].

The advantages of this study include an untargeted metabolomics approach to analyze alterations in seminal plasma metabolites, which can provide a comprehensive metabolic profile and detect the novel discovery without prior assumptions. Additionally, the longitudinal analyses linking DCAA and TCAA exposures with semen parameters (72 days interval) that cover one sperm development cycle in humans, as well as the mediating effects of seminal plasma metabolites provide human evidence for exploring potential biological mechanisms. Finally, urinary DCAA and TCAA concentrations were measured as internal biomarkers to enhance individual DBP exposure levels. However, there are also several limitations to consider. Firstly, the participants were recruited from a reproductive treatment center, which may include more men with subfertility. In addition, the included study population and the overall population exhibit differences in age, education and household income. These potentially limit the generalizability of the findings to the broader population. Nevertheless, according to the 2010 WHO normative values, 50.8% of men in our study had normal semen quality parameters, which was comparable to the 50.7% reported among 30,636 sperm donors in China's Human Sperm Bank between 2001 and 2015 [70]. Secondly, urine and semen samples were collected only once for exposure and outcome assessments, as well as metabolomic analysis, which may lead to misclassification and bias the estimates. Urinary DCAA and TCAA levels have been documented to show high within-person variability, and thus multiple urinary measures would adequately capture the long-term or cumulative DBP exposures [71]. Thirdly, urinary DCAA and TCAA levels are mainly related with the ingestion of THMs and haloacetic acids through drinking water [33,71], and their abilities to capture exposure to drinking-water DBPs through other pathways such as inhalation and dermal absorption or other unregulated/emerging DBP species like brominated and iodinated DBPs remains uncertain. Fourthly, unmeasured confounders (e.g., energy expenditure, diet and stress) or co-exposure to other environmental pollutants (e.g., air pollutants) might introduce bias into the observed associations. Further, from the exposome perspective, DBPs may interact with these co-exposure factors via shared pathways (e.g., oxidative stress) to jointly modulate seminal plasma metabolism and semen quality. Future research could consider joint exposure models. Lastly, though FDR correction was applied in the study, the two-step MWAS approach may still increase the risk of false-positive findings. Moreover, observational studies still impose inherent limitations on causal interpretation.

5. Conclusions

Our comprehensive analysis using untargeted metabolomics identified that 7 seminal plasma metabolites were associated with both urinary DCAA and TCAA concentrations and 30 metabolites were associated with all four semen quality parameters. Pathway analysis revealed that those metabolites associated with DBP exposures and semen quality parameters were both significantly enriched in the arginine and proline metabolism pathway. PI(18:1/22:6) was identified as the mediating role in the inverse relationships between urinary DCAA concentration and both sperm progressive motility and total motility. These results provide insights into the potential biomarkers of DBP exposures and semen quality and enhance the understanding of the biological mechanisms through which DBP exposures exert adverse effects on semen quality.

CRediT authorship contribution statement

Peng-Hui Liu: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Yang-Chang Zhang:** Methodology, Investigation. **Yu Miao:** Methodology, Investigation. **Min Zhang:** Methodology, Investigation. **Jin-Qin Zhu:** Methodology, Investigation. **Jia-Yue Zeng:** Investigation. **A-Xue Liu:** Investigation. **Yang-Juan Li:** Investigation. **Long Ge:** Methodology. **Xiao-Ying Liu:** Methodology, Investigation. **Yang Wu:** Methodology, Investigation. **Cheng-Ru Li:** Investigation. **Chang-Jiang Liu:** Writing – review & editing, Supervision, Conceptualization. **Sheng-Zhi Sun:** Writing – review & editing, Supervision, Conceptualization. **Qiang Zeng:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.enceco.2026.02.019.

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